

Development of a novel aortic dissection mouse model and evaluation of drug efficacy using in-vivo assays and database analyses

Yuki Izawa-Ishizawa^a, Masaki Imanishi^b, Yoshito Zamami^{b,c}, Hiroki Toya^d, Tomoko Nagao^d, Marin Morishita^e, Koichi Tsuneyama^f, Yuya Horinouchi^a, Yoshitaka Kihira^g, Kenshi Takechi^h, Yasumasa Ikeda^a, Koichiro Tsuchiya^d, Masanori Yoshizumiⁱ, Toshiaki Tamaki^a, and Keisuke Ishizawa^{b,c}

Objective: Aortic dissection is a life-threatening disease. At present, the only therapeutic strategies available are surgery and antihypertensive drugs. Moreover, the molecular mechanisms underlying the onset of aortic dissection are still unclear. We established a novel aortic dissection model in mice using pharmacologically induced endothelial dysfunction. We then used the Japanese Adverse Drug Event Report database to investigate the role of pitavastatin in preventing the onset of aortic dissection.

Methods and results: To induce endothelial dysfunction, *N* ω -nitro-L-arginine methyl ester, a nitric oxide synthase inhibitor, was administered to C57BL/6 mice. Three weeks later, angiotensin II (Ang II) and β -aminopropionitrile (BAPN), a lysyl oxidase inhibitor, were administered with osmotic mini-pumps. False lumen formation was used as the pathological determinant of aortic dissection. The incidences of aortic dissection and death from aneurysmal rupture were significantly higher in the *N* ω -nitro-L-arginine methyl ester, Ang II, and BAPN (LAB) group than they were in the Ang II and BAPN (AB) group. Pitavastatin was administered orally to LAB mice. It significantly lowered the incidences of dissection and rupture. It also decreased inflammation and medial degradation, both of which were exacerbated in the LAB group. The Japanese Adverse Drug Event Report database analysis indicated that there were 113 cases of aortic dissection out of 95 090 patients (0.12%) not receiving statins but only six cases out of 16 668 patients receiving statins (0.04%) (odds ratio: 0.30; $P=0.0043$).

Conclusion: Our results suggest that endothelial dysfunction is associated with the onset of aortic dissection and pitavastatin can help prevent this condition.

Keywords: angiotensin II, aortic dissection, endothelial dysfunction, lysyl oxidase inhibitor, nitric oxide synthase inhibitor, pitavastatin, the Japanese Adverse Drug Event Report Database

Abbreviations: AB, Ang II + BAPN treated group; Ang II, angiotensin II; BAPN, β -aminopropionitrile; CI, confidence interval; DHE, dihydroethidium; eNOS, endothelial nitric oxide synthase; EVG, Elastica van Gieson's; H-LAB, higher

dose (100 mg/kg per day) of L-NAME treated LAB group; JADER, The Japanese Adverse Drug Event Report; LAB, L-NAME + Ang II + BAPN treated group; L-LAB, lower dose (10 mg/kg per day) of L-NAME treated LAB group; L-NAME, *N* ω -nitro-L-arginine methyl ester; MCP-1, monocyte chemoattractant protein-1; NO, nitric oxide; NO_x, nitrite derivatives; ROR, reporting odds ratio; VCAM-1, vascular cell adhesion molecule-1; VE-cadherin, vascular endothelialcadherin

INTRODUCTION

Aortic dissection is a severe disease. The aortic wall separates into two layers at the medial level and creates a true lumen and a false lumen. In most cases, its onset and the death that ensues are sudden. Epidemiological investigations into aortic dissection remain inadequate. Nevertheless, recent reports have demonstrated that the actual number of patients with aortic dissection is much higher than previously estimated [1] and continues to rise [2]. However, the only available

Journal of Hypertension 2019, 37:73–83

^aDepartment of Pharmacology, Tokushima University, Graduate School of Biomedical Sciences, ^bDepartment of Pharmacy, Tokushima University Hospital, ^cDepartment of Clinical Pharmacology and Therapeutics, ^dDepartment of Medical Pharmacology, Tokushima University, Graduate School of Biomedical Sciences, ^eDepartment of Emergency Pharmaceutical Sciences, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, ^fDepartment of Pathology and Laboratory Medicine, Tokushima University, Graduate School of Biomedical Sciences, Tokushima, ^gFaculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, Fukuyama, ^hClinical Trial Center for Developmental Therapeutics, Tokushima University Hospital, Tokushima and ⁱDepartment of Pharmacology, Nara Medical University, Kashihara, Japan

Correspondence to Keisuke Ishizawa, PhD, Department of Clinical Pharmacology and Therapeutics, Tokushima University, Graduate School of Biomedical Sciences, 3-18-15, Kuramoto, Tokushima 7708503, Japan. Tel: +81 88 633 7212; fax: +81 88 633 7472; e-mail: ishizawa@tokushima-u.ac.jp

Received 4 June 2018 Accepted 11 July 2018

J Hypertens 37:73–83 Copyright © 2018 The Author(s). Published by Wolters Kluwer Health, Inc. This is an open access article distributed under the terms of the Creative Commons Attribution-Non Commercial-No Derivatives License 4.0 (CCBY-NC-ND), where it is permissible to download and share the work provided it is properly cited. The work cannot be changed in any way or used commercially without permission from the journal.

DOI:10.1097/HJH.0000000000001898

therapeutic strategies for aortic dissection are either surgery or antihypertensive agents and complete rest. Thus, the development of effective drugs for the prevention and treatment of aortic dissection is necessary. Known risk factors for aortic dissection are male sex, hypertension, aging, and certain genetic connective tissue disorders [1]. Hypertension and medial fragility have been reported as the most important pathophysiological conditions contributing to aortic dissection and aneurysm [3]. The administration of angiotensin II (Ang II) and β -aminopropionitrile (BAPN) to mice pharmacologically induces acute aortic aneurysm by causing hypertension and medial degradation [4]. However, the actual mechanism underlying the onset of aortic aneurysm or dissection is still unclear.

Recently, the significance of endothelial dysfunction during the onset of aortic dissection has been addressed. Fan *et al.* [5] demonstrated that cell-specific endothelial NADPH oxidase (NOX) 2 overexpression increases aortic dissection susceptibility. Gavazzi *et al.* [6] also reported that NOX1 is involved in Ang II-induced aortic dissection. Clinical studies have shown that endothelial nitric oxide (NO) synthase (eNOS) gene polymorphism is associated with aortic dissection [7]. These findings indicate that endothelial dysfunction may be a prerequisite for the onset of acute aortic dissection and NOS participates in its pathogenesis. *N* ω -nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor, is often used to induce endothelial dysfunction and hypertension in mice [8,9]. In the present study, we tested the hypothesis that adding L-NAME to Ang II and BAPN could accelerate the onset of dissection but not aneurysm.

Statins are 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors. They are widely used as lipid-lowering agents. To date, many studies show that, because of their pleiotropic effects, statins are effective in preventing cardiovascular diseases. In a previous study, we demonstrated that pitavastatin protects the endothelium by increasing eNOS expression [10]. The effect of statins in delaying the progression of aortic aneurysm has been suggested based on retrospective clinical studies [11]. In the present study, we used a novel mouse model and data mining techniques to evaluate the efficacy of pitavastatin in reducing the incidence of aortic dissection related to endothelial dysfunction.

MATERIALS AND METHODS

Ethics statement

The current study conformed to the Guide for the Care and Use of Laboratory Animals [12]. All animal procedures were performed in accordance with the guidelines of the Animal Research Committee of the University of Tokushima Graduate School. The protocols were approved by the Tokushima University Institutional Review Board for Animal Protection.

Reagents

Ang II, BAPN, and L-NAME were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Pitavastatin was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All other reagents and instruments used in the present study are commercially available.

Mice and in-vivo experimental strategies

Male C57BL/6J mice (10–12 weeks; 25–30 g) were purchased from CLEA Japan Inc. (Tokyo, Japan). One hundred and sixty mice were used in all sequential experiments of this study. This sample size was statistically estimated using G*power software (Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany). The mice were divided into four different groups, as described below. They were housed in a temperature-controlled room at 25 °C under a 12-h light/dark cycle. They had free access to food and water. L-NAME (10 or 100 mg/kg per day) was orally administered in drinking water from age 7 weeks until the end of the experiment. Dosages were empirically determined in a previous study [13]. After 3 weeks, the mice were implanted dorsally with two subcutaneous osmotic mini-pumps (Model 1002 Micro-osmotic Pump; Alzet, Cupertino, California, USA) to administer Ang II and BAPN. Prior to the surgery, the mice were anesthetized by intraperitoneal injection of 100–150 mg/kg sodium pentobarbital. Pedal withdrawal reflex, toe pinch reflex, muscle relaxation, and respiratory rates were monitored to ensure that the anesthesia was adequate. The dosage was increased if the mice moved in response to pain after 30 min from the start of the operation. Control mice were sham-operated. Ang II dissolved in normal saline was continuously infused at 1000 ng/kg per day for 1 or 6 weeks either to perform all assays including tissue histopathology or to estimate survival rates, respectively. BAPN (150 mg/kg per day) was dissolved in saline and administered by osmotic mini-pump at the same time as Ang II loading and was maintained for 1–2 weeks. The groups were designated as follows:

- (1) Control: untreated group
- (2) AB: Ang II + BAPN-treated group
- (3) L-LAB: lower L-NAME dose (10 mg/kg per day) + Ang II + BAPN-treated group
- (4) H-LAB: higher L-NAME dose (100 mg/kg per day) + Ang II + BAPN-treated group

Pitavastatin was administered via feeding needle at the rate of 3 mg/kg per day from age 7 weeks until the end of the experiment. Dosages were empirically determined in a previous study [14]. Oral saline was administered to the control animals.

Systolic blood pressure

SBP was measured in conscious mice by tail-cuff plethysmography (BP-98A; Softron, Tokyo, Japan).

Isolation of mouse aortas and measurement of aortic diameters

At the end of the experiment, the animals were anesthetized by intraperitoneal sodium pentobarbital injection (≥ 150 mg/kg) and euthanized by cervical dislocation. The whole hearts and aortas were isolated and photographed under a SZ61 Olympus stereomicroscope (Olympus Corp., Tokyo, Japan). Maximum aortic diameters were measured with ImageJ v. 1.37 (National Institutes of Health, Bethesda, Maryland, USA).

Histology and immunohistochemistry

For the morphometry, the aortas were resected and placed in 10% buffered formalin. After fixation, the tissues were embedded in paraffin. Sections (5 μm thick) were stained with hematoxylin/eosin or Elastica van Gieson's (EVG) stain. Immunohistochemistry was performed using vascular cell adhesion molecule-1 (VCAM-1) antibody (1 : 1000 dilution) and a Vectastain ABC Kit (Funakoshi, Tokyo, Japan). Measurements were taken with ImageJ v. 1.37 (National Institutes of Health).

Quantitative reverse transcription-PCR

The mRNA expression levels of CD68, F4/80, TNF- α , and monocyte chemoattractant protein (MCP)-1 in the aortas were analyzed by quantitative reverse transcription-PCR as previously described [15]. Sequences of the amplification primer pairs are as below (5'-3'): CD68; forward-CTTCCCACAGGCAGCACAG, reverse-AATGATGAGAGG-CAGCAAGAGG, F4/80; forward-CTTGGCTATGGGCTT-CCAGTC, reverse-GCAAGGAGGACAGAGTTTATCGTG, TNF- α ; forward-CCAGACCCTCACACTCAGATC, reverse-CACTTGGTGGTTTGCTACGAC, MCP-1; forward-CCCA-ATGAGTAGGCTGGAGA, reverse-TCTGGACCCATTCTCT-CTTG.

Nitrite/nitrate (NO_x) measurements

The total amounts of NO_2 and NO_3 in the aortas were measured by the Griess method using an NO_2/NO_3 Assay Kit (Dojindo Laboratories, Kumamoto, Japan). Briefly, the samples were incubated in equal volumes of Griess reagent [2% sulfanilamide and 0.1% *N*-(1-naphthyl)ethylenediamine] for 15 min followed by incubation in vanadium(III) chloride for 5 h. The absorbance was measured at 540 nm using a FilterMax F3 microplate reader (Molecular Devices, San Jose, California, USA). Values were calculated using NO_2 and NO_3 standards.

Reactive oxygen species detection in the aortas

Dihydroethidium (DHE) (10 $\mu\text{mol/l}$) (Dojindo Laboratories) was used to measure the in-situ production of superoxide O_2^- in cryosections of the mouse aortas as previously described [15]. The intensity of the fluorescence was analyzed and quantified with ImageJ v. 1.37 (National Institutes of Health).

En face immunostaining

After 3 weeks of L-NAME and pitavastatin administration, the aortas of 10-week mice were subjected to en-face staining. Briefly, the mice were anesthetized with pentobarbital and euthanized by opening the thoracic cavity. The arterial tree was perfused with heparinized saline (40 USP U/ml) starting at the left ventricle, and then with 4% paraformaldehyde in PBS for 10 min. The formalin-fixed aortas were excised and the adipose tissue surrounding them was removed. They were cut open longitudinally, permeabilized with PBS containing 0.1% Triton X-100, and blocked with PBS containing 10% goat serum and 2.5% Tween 20 for 30 min. The aortas were incubated with serum-free protein blocking buffer (#X0909; Dako Denmark A/S, Glostrup, Denmark) for 30 min, followed

by overnight incubation with rat antivascular endothelial-cadherin (VE-cadherin) (7 $\mu\text{g/ml}$) in antibody diluent buffer (#S0809; Dako Denmark A/S). After a PBS rinse, antirat IgG (1 : 1000 dilution) (Alexa Fluor 488; Invitrogen, Carlsbad, California, USA) was applied for 1 h at room temperature (16–25 $^{\circ}\text{C}$). The samples were analyzed with an A1R laser scanning confocal microscope (Nikon Instech Co. Ltd., Tokyo, Japan). Ten to 15 optical sections were collected at 0.3–0.5- μm increments. A z-stack of sections was also obtained, consisting of $\sim 4\text{-}\mu\text{m}$ -thick sections from the luminal surface.

Vascular permeability

Ten-week-old mice either untreated or treated with 10 mg/kg per day L-NAME for 3 weeks were injected with 100 μl Evans blue (1% solution in PBS) via the tail vein 30 min before euthanasia. They were then transcardially perfused with heparinized PBS to remove intravascular dye. The gross coloring of the isolated aortas was observed. The aortas were then flash-frozen and sectioned into 10- μm slices. Evans blue dye extravasation was observed under a fluorescence microscope (ECLIPSE Ti-U, Nikon, Tokyo, Japan).

Cell culture

Human aortic endothelial cells were purchased from TaKaRa Bio Inc., Kusatsu, Shiga, Japan. They were cultured in Endothelial Cell Growth Medium 2 (TaKaRa Bio Inc.). After overnight serum starvation, passage four to eight cells were treated either with pitavastatin (10 or 100 nmol/l), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide sodium (carboxy-PTIO) (10 or 100 $\mu\text{mol/l}$) (Dojindo Laboratories), or 1-hydroxy-2-oxo-3,3-bis(2-aminoethyl)-1-triazene (NOC18) (10 or 100 $\mu\text{mol/l}$) (Dojindo Laboratories) for 24 h. Cell lysates were subjected to western blotting as described in western blot section.

Western blot

The proteins from the mouse aortas and cultured cells were analyzed by western blotting as described previously [15]. The antibodies detected eNOS (Cell Signaling Technology Inc., Danvers, Massachusetts, USA), VE-cadherin (Cell Signaling Technology Inc.), and β -actin (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA), all at 1 : 1000 dilution.

Statistical analysis

Data are presented as means \pm standard error. Statistical analysis was performed using StatMate IV for Windows (ATMS Co. Ltd., Tokyo, Japan). Normally distributed continuous variables for more than three groups were compared with two-way analysis of variance. Two-group comparisons were made with Student's *t* tests. Nonnormally distributed continuous variables were compared with the Kruskal–Wallis test. Other pairwise comparisons were made with the Mann–Whitney *U* test. Nominal categorical data between groups were compared with the χ^2 -test or Fisher's exact test where appropriate. For all statistical tests, *P* less than 0.05 and 0.001 were considered to indicate significant and highly significant differences, respectively.

Analysis of the Japanese Adverse Drug Event Report database

Adverse event data recorded in the Japanese Adverse Drug Event Report (JADER) database from April 2004 until April 2015 were analyzed. They were obtained from the Pharmaceuticals and Medical Devices Agency (<http://www.pmda.go.jp>). The JADER database structure complies with the international safety reporting guidelines of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH E2B). The database consists of patient demographic information (demo), drug information (drug), adverse events (reac), and primary illness (hist). The JADER database does not include case report identification codes (A1.11). Therefore, no data cleaning was required. The 'Drug' file (drug information) contains the role code assigned to each drug, namely suspected drug, interacting drug, and concomitant drug. In this study, only the records for suspected drugs were analyzed.

The drug class selected for this investigation was the statins (atorvastatin, fluvastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin). All reported adverse events of interest (i.e., aortic dissection) were defined as 'cases' and all other reported adverse events were designated 'noncases.' The adverse event risk was evaluated with the reporting odds ratio (ROR) calculated using a case/noncase method [16,17]. A 2×2 contingency table was prepared and used as the framework for the analysis

(Supplementary Table 1, <http://links.lww.com/HJH/A991>). The effects on aortic dissection of combining statins with the suspected drug were analyzed. At least three cases were reported, and the lower limit of the 95% confidence interval (CI) of the ROR was more than 1.

RESULTS

Administration of *N* ω -nitro-L-arginine methyl ester to the angiotensin II + β -aminopropionitrile aortic aneurysm model triggered the onset of aortic dissection

L-NAME administration started 3 weeks before Ang II + BAPN was used to establish an aortic aneurysm model (western blot section). Ang II + BAPN treatments were continued for 6 weeks to investigate the incidence and survival rate of aortic dissection. The low-dose (10 mg/kg per day) L-NAME changed neither body weight nor SBP relative to that of the AB group (Fig. 1a and b). A Kaplan–Meier analysis revealed no significant difference between the AB and L-LAB groups in terms of overall survival rate. The incidence of death from aortic rupture or dissection in the L-LAB group increased within 1 week after treatment with Ang II + BAPN (Fig. 1c). The L-LAB group showed significantly higher incidences of aortic dissection [44% (11/25); $P < 0.01$] and rupture [36% (9/25); $P < 0.05$] than did the AB group [8 (4/51) and 16% (8/51), respectively] after 6 weeks of treatment (Table 1).

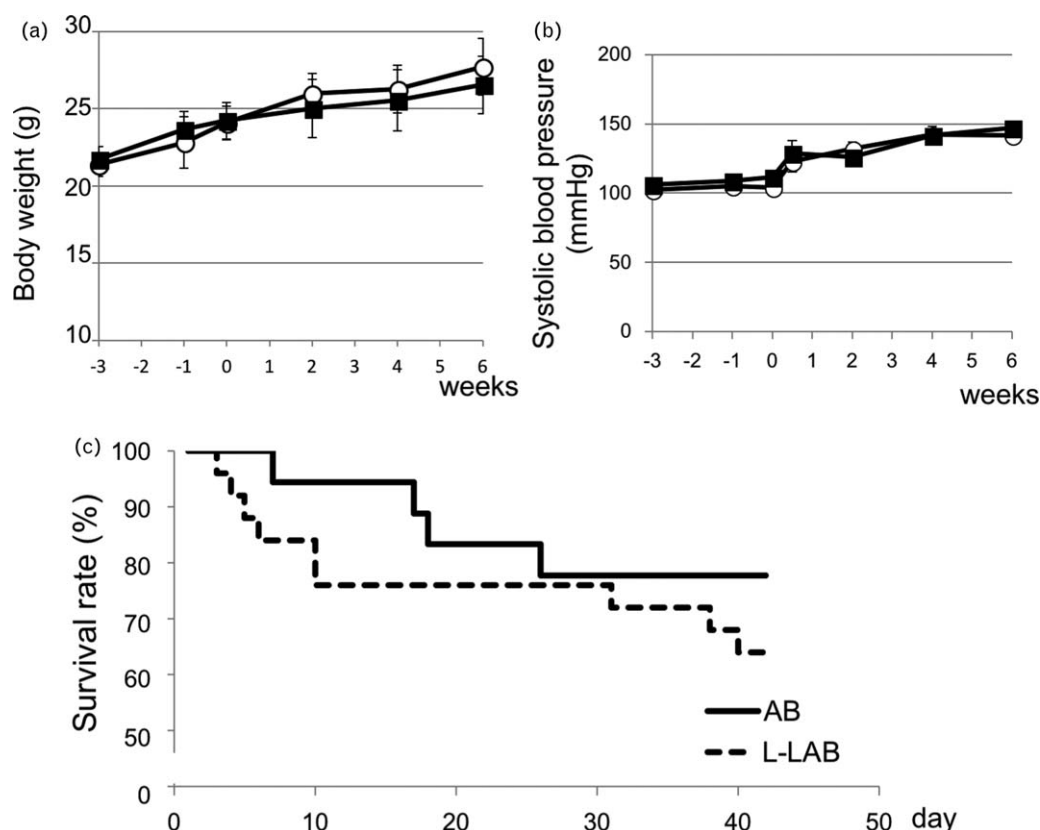


FIGURE 1 Effects of *N* ω -nitro-L-arginine methyl ester addition to aneurysm model. Week 0 or Day 0 indicates starting time of angiotensin II + β -aminopropionitrile loading. Mice were monitored until Week 6. Body weight (a) and SBP (b) are expressed as means \pm standard error (open circle: AB group; black square: L-LAB group). Survival rates were calculated by the Kaplan–Meier method (c). Values were statistically analyzed using Student's *t* test (a and b) or the generalized Wilcoxon signed rank test (c). $n = 19$ –51 for each group. * $P < 0.05$, ** $P < 0.01$ vs. AB group. AB, Ang II + BAPN-treated group; L-LAB, 10 mg/kg per day L-NAME in Ang II + BAPN-treated group.

TABLE 1. Incidences of aneurysm, dissection, and rupture after 6 weeks observation or at the time of death

	Control, <i>n</i> (%)	AB, <i>n</i> (%)	L-LAB, <i>n</i> (%)
TAA	0/19 (0)	19/51 (37)	13/25 (52)
AAA	0/19 (0)	28/51 (55)	19/25 (76)
AD	0/19 (0)	4/51 (8)	11/25 (44)*
Rupture	0/19 (0)	8/51 (16)	9/25 (36)**

The incidence of TAA, AAA, AD, and rupture were shown. Values are shown as *n* (%). Data are statistically analyzed by chi-squared test. AAA, abdominal aortic aneurysm; AB, Ang II + BAPN treated group; AD, aortic dissection; L-LAB, 10 mg/kg per day of L-NAME + Ang II + BAPN treated group; TAA, thoracic aortic aneurysm.

**P* < 0.01 vs. AB group.

***P* < 0.05.

The Japanese Adverse Drug Event Report database showed a relatively lower incidence of aortic dissection with statin administration

The JADER database enables us to find candidate drugs that can prevent aortic dissection during drug therapy. To determine whether statins decreased the incidence of drug-induced aortic dissection, we used the JADER database to analyze the effect of combining statins with the candidate drug.

Forty-four candidate drugs for aortic dissection therapy were selected from the JADER database. For each drug, at least three cases were reported with a lower limit of the 95% CI for ROR more than 1 (Supplementary Table 2, <http://links.lww.com/HJH/A991>). The incidence rates were compared with or without the concomitant use of pitavastatin or at least one other statin. As shown in Table 2, the incidences of aortic dissection with and without pitavastatin coadministration were 0.06 and 0.11%, respectively. The odds ratio (OR) was 0.52 (95% CI, 0.07–3.72; *P* = 0.5148). Coadministration with any statin decreased the incidence to 0.04%. In contrast, the incidence was 0.12% without the use of statins (OR = 0.30; 95% CI, 0.13–0.69; *P* = 0.0043). Therefore, there was a significant decrease in the incidence of aortic dissection with statin administration.

Pitavastatin prevented the incidence of aortic dissection in LAB mice

To estimate whether LAB-induced aortic dissection in mice was useful for evaluating drug efficacy, we examined the effects of pitavastatin on aortic dissection. Representative photomicrographs of the overall appearance and EVG staining of the aortas are shown in Fig. 2a and b, respectively. Photomicrograph measurements indicated that pitavastatin inhibited increases in the maximum diameters of the thoracic and abdominal aortas of the L-LAB mice (Fig. 2c

and d). The incidence of aortic dissection increased to similar levels in both the L-LAB (70%, 7/10) and H-LAB (62%, 8/13) groups. Pitavastatin administration delayed the onset of aortic dissection in both the L-LAB (18%, 2/11) and H-LAB (14%, 2/14) groups to levels comparable with that of the AB group (Table 3). The degree of medial elastic fiber disruption was quantified by EVG staining (Fig. 2b). The L-LAB and H-LAB groups presented with more elastic fiber disruption than the AB group did. Pitavastatin reduced medial elastic fiber disruption to the level of the AB group (Fig. 2e).

Figure 2g shows that 3-week administration of 100 mg/kg per day L-NAME without Ang II loading elevated SBP. However, the blood pressures (BPs) in all groups, except for the control, were similar after Ang II loading. Therefore, pitavastatin may not have reduced the incidence of aortic dissection by lowering BP.

Inflammatory responses were investigated in the LAB model. The administration of L-NAME to AB mice increased the expression of the *CD68* and *F4/80* genes (Fig. 3a and b). These are markers of macrophages that had infiltrated the vascular walls, which were also observed by Mac-2 immunostaining (Fig. 3c). These increase in macrophage infiltration were suppressed by pitavastatin treatment in the L-LAB model. Pitavastatin also significantly suppressed the upregulation of other proinflammatory genes in the aortas of the L-LAB model, including TNF- α , MCP-1, and VCAM-1 (Fig. 3d–f).

ω -nitro-L-arginine methyl ester pretreatment for 3 weeks induced endothelial cell injury

To determine whether pitavastatin attenuates L-NAME-induced endothelial dysfunction, mice were either untreated or subjected to L-NAME + pitavastatin treatment for 3 weeks before starting AB administration. The administration of L-NAME for 3 weeks decreased aortic eNOS protein expression (Fig. 4a) and decreased NO_x production. NO_x significantly increased in response to pitavastatin administration (Fig. 4b). DHE staining revealed that oxidative stress was increased by the administration of 10 or 100 mg/kg per day L-NAME and was suppressed by pitavastatin (Fig. 4c and d).

It was recently reported that the expression of VE-cadherin modulates vascular permeability with NO [18]. En-face staining and western blotting revealed that VE-cadherin protein expression was decreased in the aortas of mice treated with L-NAME (Fig. 5a and b). Evans blue dye leakage showed that L-NAME treatment induced vascular hyperpermeability (Fig. 5c and d), possibly impairing the integrity of endothelial cell junctions.

TABLE 2. Decrease in the occurrence of suspected drug-induced aortic dissection by drug A in the Japanese Adverse Drug Event Report database

Drug A	Cases without drug A, <i>n</i> (%)	Cases with drug A, <i>n</i> (%)	Odds ratio (95% CI)	<i>P</i>
Pitavastatin	118/109966 (0.11)	1/1792 (0.06)	0.52 (0.07–3.72)	0.5148
All statins	113/95090 (0.12)	6/16668 (0.04)	0.30 (0.13–0.69)	0.0043

CI, confidence interval.

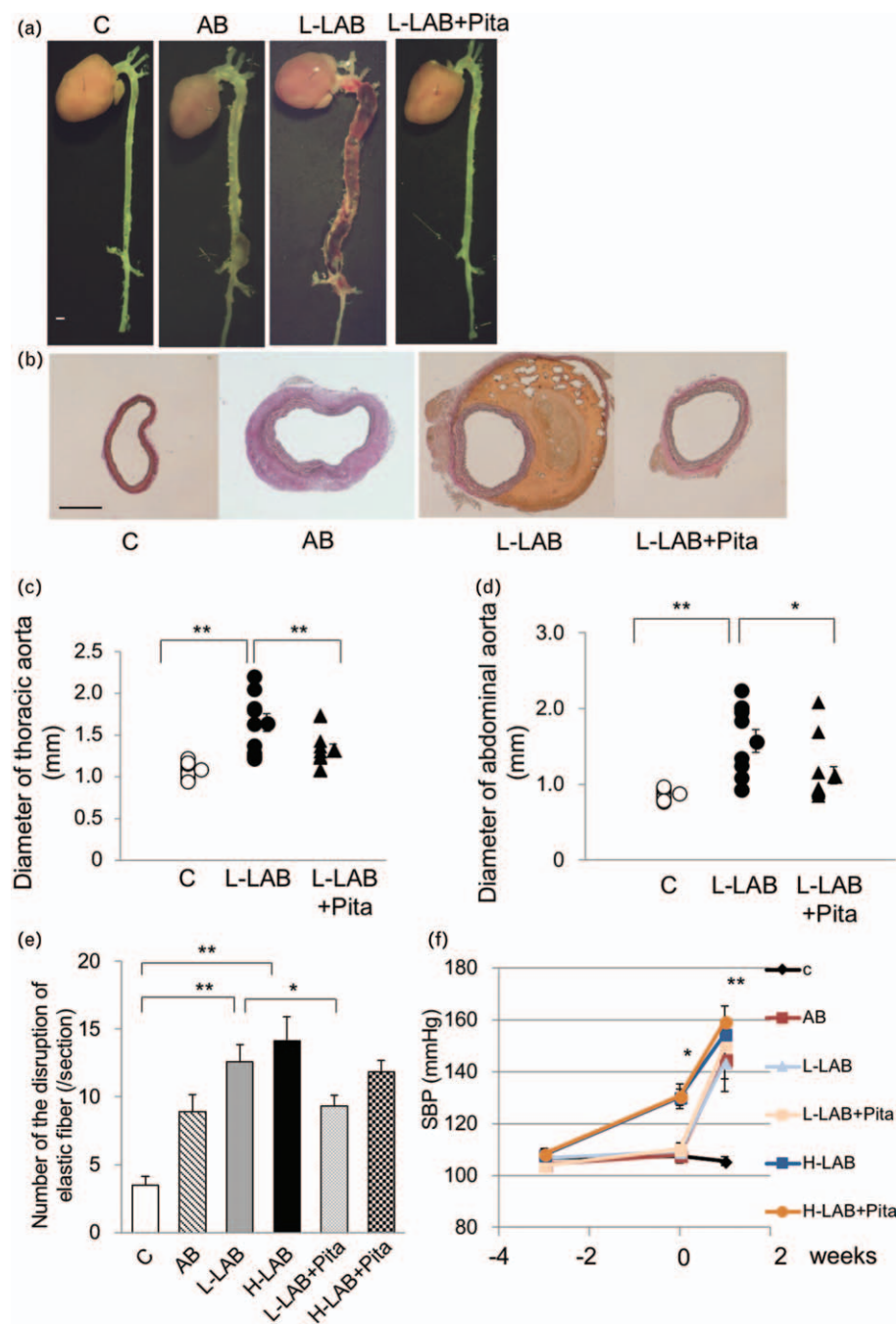


FIGURE 2 Effect of pitavastatin on aortic dissection model (LAB). Representative images of gross aortas (a; bar: 0.5 mm) and Elastic van Gieson's staining (b; bar: 0.5 mm). Diameters of thoracic aortas (c) and abdominal aortas (d) were measured. Numbers of disrupted elastic fibers observed with Elastic van Gieson's staining (e). SBP was monitored from the onset of *N* ω -nitro-L-arginine methyl ester administration until 1 week after angiotensin II + β -aminopropionitrile loading (f). *n* = 5–11. Values were analyzed using two-way analysis of variance for repeated measures (c–f). **P* < 0.05, ***P* < 0.01 vs. control. L-LAB, LAB group treated with lower L-NAME dose (10 mg/kg per day); H-LAB, LAB group treated with higher L-NAME dose (100 mg/kg per day).

TABLE 3. Incidence of aortic dissection and rupture at Day 7 after the initiation of angiotensin II + β -aminopropionitrile loading		
	AD, <i>n</i> (%)	Rupture, <i>n</i> (%)
AB	2/10 (20)	1/10 (10)
L-LAB	7/10 (70)	2/10 (20)
H-LAB	8/13 (62)	3/13 (23)
L-LAB + Pita	2/11 (18)*	0/11 (0)
H-LAB + Pita	2/14 (14)*	1/14 (7)

Values are shown as *n* (%). Data are statistically analyzed by chi-squared test. AB, Ang II + BAPN treated group; H-LAB, 100 mg/kg per day of L-NAME + Ang II + BAPN treated group; L-LAB, 10 mg/kg per day of L-NAME + Ang II + BAPN treated group; Pita, pitavastatin. **P* < 0.05 vs. without pitavastatin treatment.

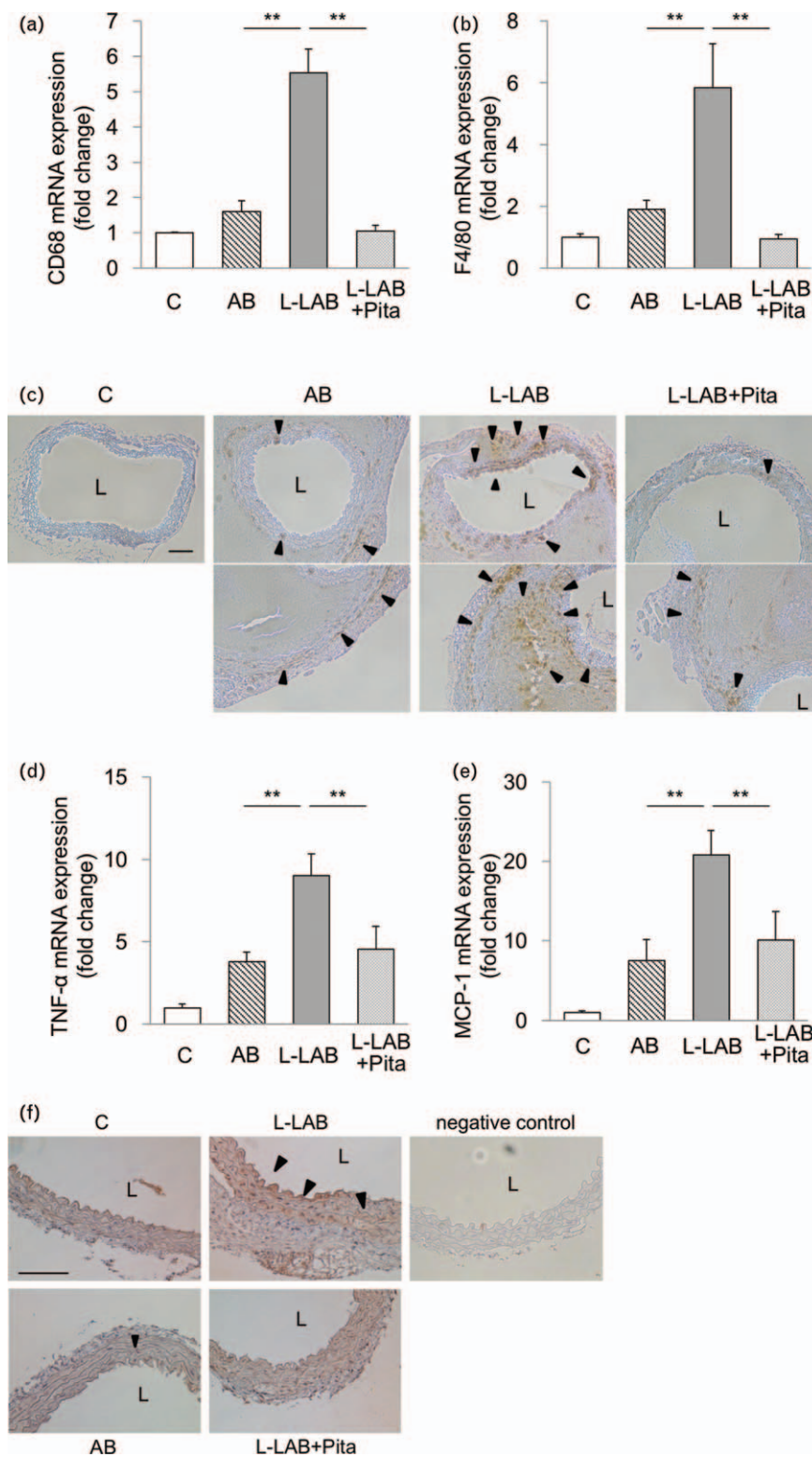


FIGURE 3 Effects of pitavastatin on aortic inflammatory responses. Graphs show mRNA expressions of aortic inflammatory markers CD68 (a), F4/80 (b), TNF- α (d), and monocyte chemoattractant protein-1 (e). $n = 5$. Values are fold increases relative to the average of the control and are expressed as means \pm standard error. Two-way analysis of variance for repeated measures was performed along with Bonferroni's post-hoc tests. $^{*}P < 0.01$. Panels (c) and (f) show representative images of Mac-2 (c) and vascular cell adhesion molecule-1 (f) expressions. Arrowheads point to positive staining. 'L' = lumen side. Bar: 0.1 mm.

To verify whether NO modulates VE-cadherin expression, cultured endothelial cells were exposed either to the NO scavenger, carboxy-PTIO, or to the NO donor, NOC18. NO production varied directly with VE-cadherin expression

and vice versa (Fig. 5e). Pitavastatin increased eNOS expression in cultured endothelial cells. This observation corroborated those of earlier studies [18]. VE-cadherin protein expression increased in response to pitavastatin

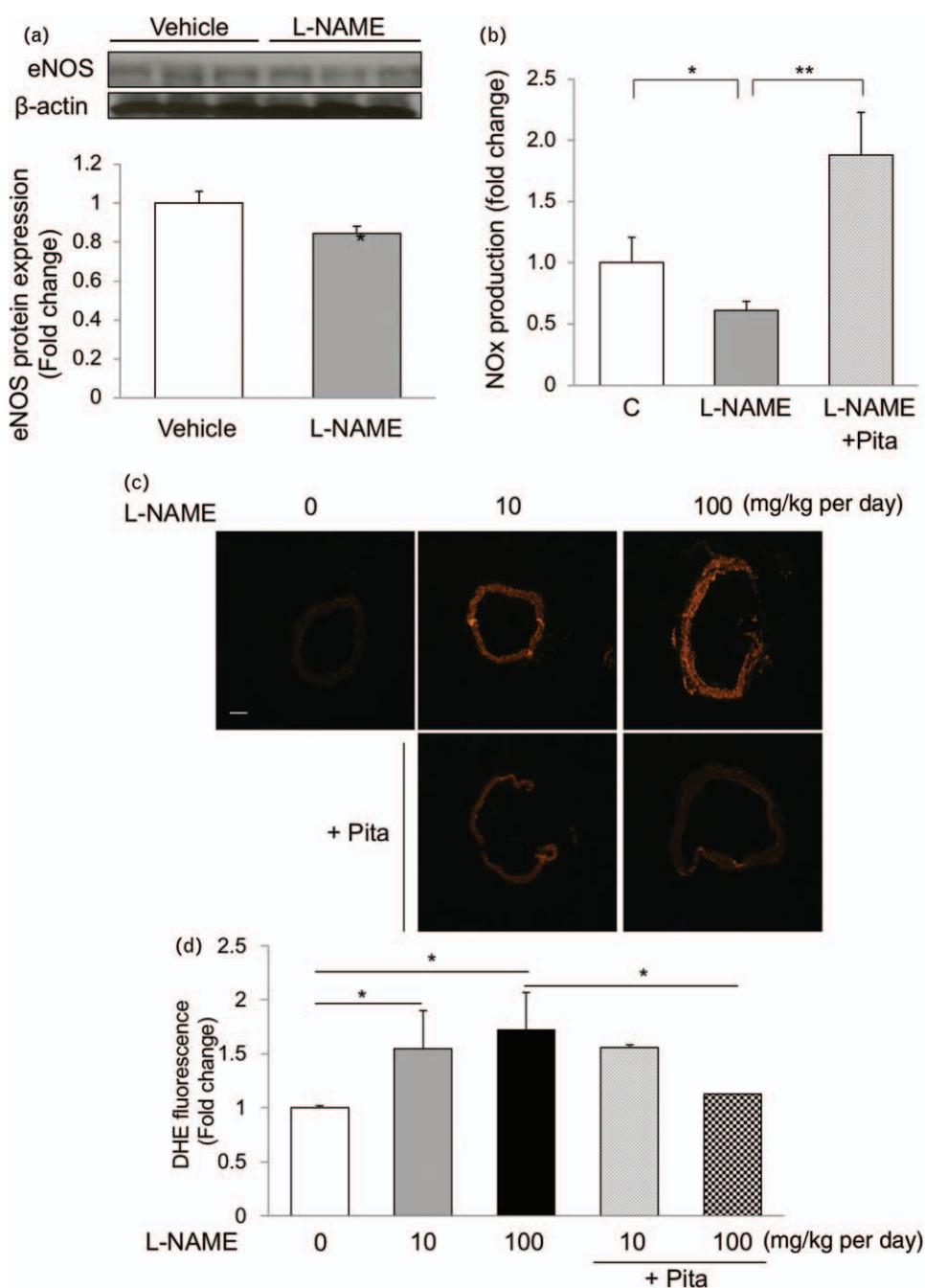


FIGURE 4 Effects of pitavastatin on oxidative stress in aortas. Aortic endothelial nitric oxide synthase protein expression (a) and nitrite/nitrate (NO_x) production (b) after 3 weeks treatment with *N* ω -nitro-L-arginine methyl ester (10 mg/kg per day) were determined by western blotting and Griess method, respectively. Oxidative stress was estimated by dihydroethidium staining. (c) Representative images; bar: 0.2 mm. (d) Quantified dihydroethidium fluorescence. $n=4-7$. Values are fold increases relative to the average of the control and are expressed as means \pm standard error. Two-way analysis of variance for repeated measures was performed along with Bonferroni's post-hoc tests. * $P<0.05$, ** $P<0.01$.

treatment (Fig. 5f and g). Pitavastatin may attenuate endothelial cell junction failure by increasing eNOS and VE-cadherin expression.

DISCUSSION

Several investigators have attempted to establish an animal model in which a high rate of aortic dissection could be induced. The most successful to date has been a mouse

model with BAPN/Ang II [19]. In earlier studies, BAPN was administered at higher doses and for longer durations than in our study, causing acute aortic dissection in 100% of the mice within 24 h of the start of Ang II loading. In previous models, aortic dissection was almost completely suppressed in matrix metalloproteinase-9-deficient mice [19]. Therefore, the aortic dissection induced in earlier models may have been the result of medial fragility and degradation. In fact, aortic dissection is known to occur in Marfan

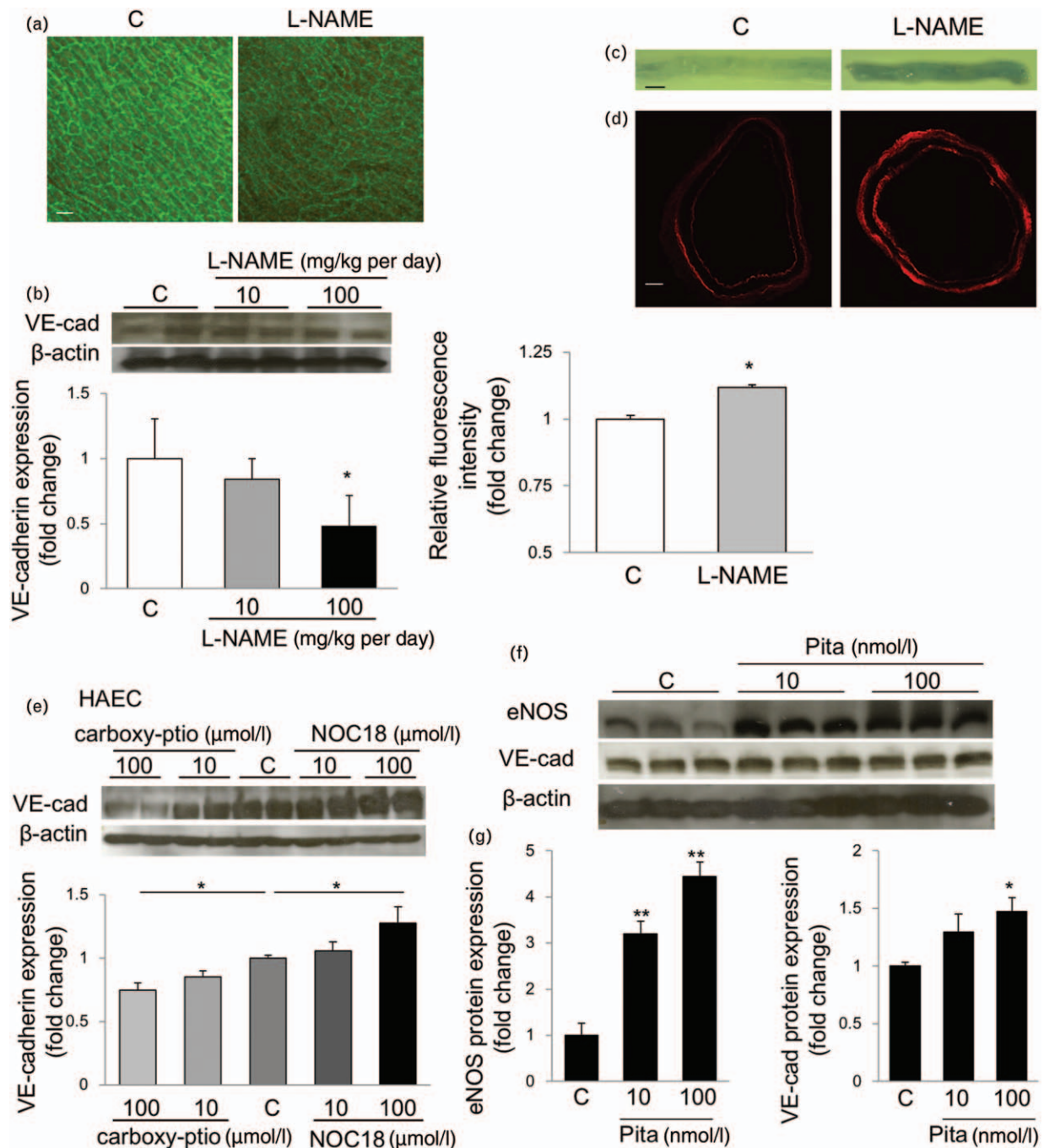


FIGURE 5 *N* ω -Nitro-L-arginine methyl ester-impaired mouse aortic endothelial cell junctions. Mice were treated with or without 100 mg/kg per day *N* ω -nitro-L-arginine methyl ester for 3 weeks. Aortas were subjected to en-face staining for vascular endothelialcadherin (a, green; bar: 50 μ m) or western blotting [(b1) representative blots and (b2) quantified intensity]. Evans blue stain leakage was observed in mice treated with or without 100 mg/kg per day *N* ω -nitro-L-arginine methyl ester for 3 weeks [(c) low magnification pictures; bar: 1 mm, (d1) Evans blue fluorescence; bar: 0.1 mm, and (d2) quantified fluorescence intensity]. Vascular endothelialcadherin expression in cultured endothelial cells measured by western blotting. Human aortic endothelial cells (e) treated with either carboxy-PTIO or NOC18 for 48 h. Panel (e1) shows representative blots. Panel (e2) shows intensity. Human aortic endothelial cells were also stimulated with pitavastatin for 48 h. Protein expressions of endothelial nitric oxide synthase and vascular endothelialcadherin were analyzed by western blotting. Representative bands (f) and quantified intensity (g) are shown. $n = 4-8$. Values are fold increases relative to the average of the control and are expressed as means \pm standard error. Two-way ANOVA for repeated measures was performed along with Bonferroni's post-hoc tests. * P < 0.05, ** P < 0.01 vs. control.

syndrome and other connective tissue disorders. However, we deemed it necessary to develop an alternative animal model simulating complications of metabolic syndrome, aging, and other disorders rather than specific genetic conditions. Therefore, we administered BAPN at a dose just high enough to induce aneurysm. We added L-NAME

pretreatment instead of increasing BAPN dose. L-NAME was originally used in hypertension models. Nevertheless, it has been reported to cause endothelial dysfunction by suppressing NO independently of hypertension [20,21]. NO is essential for maintaining endothelial functions, such as vasodilatation, vascular regeneration, inhibition of smooth

muscle cell proliferation and migration, platelet adherence, and leukocyte chemotaxis [22,23]. Therefore, L-NAME-induced NO suppression causes severe endothelial dysfunction and damage [24]. Even low doses of L-NAME increased inflammation and hyperpermeability leading to the early onset of aortic dissection without a significant elevation in BP relative to the AB group. L-NAME-induced endothelial dysfunction may trigger aortic dissection regardless of the impairment of endothelium-dependent vasodilatation. Our model also showed isolated endothelial dysfunction without significant SBP elevation. SBP rose soon after the initiation of Ang II treatment (Fig. 1). Under L-NAME treatment, aortic dissection occurred simultaneously. These results suggest that BP increase is necessary to trigger the onset of both aortic dissection and aneurysm. However, endothelial dysfunction may be the link between these two events.

Several studies demonstrated that statins prevent cardiovascular diseases by protecting endothelia [25]. Recently, we reported that pitavastatin protects against acute cardiac allograft rejection by activating endothelial extracellular-signal-regulated kinase 5 [10]. As shown in Figs. 2 and 3, pitavastatin may also prevent the onset of aortic dissection, medial degradation, and inflammation. In the present study, however, pitavastatin did not lower SBP elevation induced by high L-NAME doses, despite increasing eNOS expression and NO_x production. Perez-Guerrero *et al.* [26] showed that simvastatin restored NO_x production that had been inhibited by L-NAME but it did not lower SBP. It has been proposed that deficient eNOS-dependent vascular relaxation accounts for the L-NAME-induced hypertension model. According to our report and several others, eNOS dysfunction and NO deficiency alone may not fully explain L-NAME-induced or endothelial dysfunction-dependent hypertension [10,24–26]. Further studies are needed to elucidate the roles of eNOS-NO and the endothelium in BP regulation. Nevertheless, we can assume that the protective effects of statins against cardiovascular disease are mediated by increases in NO, independent of BP reduction.

It has been reported that tight junctions and VE-cadherin localization, phosphorylation, and expression play important roles in vascular endothelial permeability [27,28]. In our LAB model, VE-cadherin expression was diminished and vascular leakage occurred (Fig. 5). It was suggested that a possible mechanism for the protective effect of statins against aortic dissection is their impact on endothelial cell–cell junctions and changes in vascular permeability (Fig. 5). Recently, Suzuki *et al.* [29] reported that pitavastatin mitigates tight junction protein dysfunction in endothelial cells. Berry *et al.* [30] reported a correlation between intercellular junctions and aortic dissection. However, the relevance of this association is not yet fully understood. However, it may partially account for the mechanism by which pitavastatin inhibited aortic dissection onset in our model.

JADER is a large database of adverse events and useful tools for correlating preventive drug use with drug-induced adverse events [16]. We found 44 drugs in JADER associated with an increased incidence of aortic dissection. We also found that combining these drugs with statins significantly decreased the occurrence of adverse events related to aortic

dissection (Table 2 and Supplementary Table 2, <http://links.lww.com/HJH/A991>). These clinical data strongly support the findings of pharmacological experiments *in vivo* and *in vitro*.

In the present study, we established a novel murine model for aortic dissection. In LAB mice, L-NAME induced endothelial dysfunction. Compared with that of the other groups, LAB presented with more severe aortic inflammation, medial degradation, and hyperpermeability. In LAB mice, pitavastatin treatment decreased the incidence of aortic dissection and death from aortic rupture. It is believed that pitavastatin accomplished this by decreasing reactive oxygen species and increasing NO_x production and by upregulating eNOS and VE-cadherin expression. Moreover, JADER database mining suggested that pitavastatin treatment clinically prevents aortic dissection.

Our findings expand the understanding of aortic dissection. First, endothelial dysfunction is key in elucidating the pathogenesis of aortic dissection onset. Second, our novel aortic dissection model does not involve genetically engineered mice and may help identify the molecules and mechanisms by which the endothelium and medial layer contribute to the onset of this condition. Finally, this mouse model, whether alone or in combination with database analyses, could evaluate drug efficacy for aortic dissection treatment or prevention.

ACKNOWLEDGEMENTS

We acknowledge expert assistance by Support Center for Advanced Medical Sciences, Institute of Biomedical Sciences, Tokushima University Graduate School. English writing support was provided by Editage of Cactus Communications K.K. (Tokyo, Japan).

The work was supported by grants from JSPS KAKENHI grant number JP26860172, JP16K08549 (Y.I.-I.), JP15K07967 (K.I.), and the Naito Foundation (Y.I.-I.).

Previous presentations: The part of this study was presented at the international meetings as ISA2015 7th International Symposium on Atherosclerosis and the 9th International Conference on the Biology, Chemistry, and Therapeutic Applications of Nitric Oxide.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

- Howard DP, Banerjee A, Fairhead JF, Perkins J, Silver LE, Rothwell PM, *et al.* Population-based study of incidence and outcome of acute aortic dissection and premorbid risk factor control: 10-year results from the Oxford Vascular Study. *Circulation* 2013; 127:2031–2037.
- Pacini D, Di Marco L, Fortuna D, Belotti LM, Gabbieri D, Zussa C, *et al.* Acute aortic dissection: epidemiology and outcomes. *Int J Cardiol* 2013; 167:2806–2812.
- Shen YH, Zhang L, Ren P, Nguyen MT, Zou S, Wu D, *et al.* AKT2 confers protection against aortic aneurysms and dissections. *Circ Res* 2013; 112:618–632.
- Kanematsu Y, Kanematsu M, Kurihara C, Tsou TL, Nuki Y, Liang EI, *et al.* Pharmacologically induced thoracic and abdominal aortic aneurysms in mice. *Hypertension* 2010; 55:1267–1274.
- Fan LM, Douglas G, Bendall JK, McNeill E, Crabtree MJ, Hale AB, *et al.* Endothelial cell-specific reactive oxygen species production increases susceptibility to aortic dissection. *Circulation* 2014; 129:2661–2672.

6. Gavazzi G, Deffert C, Trocme C, Schappi M, Herrmann FR, Krause KH. NOX1 deficiency protects from aortic dissection in response to angiotensin II. *Hypertension* 2007; 50:189–196.
7. Ekmekci A, Uluganyan M, Gu Ngor B, Abaci N, Ozcan KS, Ertas G, *et al.* Association between endothelial nitric oxide synthase intron 4a/b polymorphism and aortic dissection. *Türk Kardiyol Dern Ars* 2014; 42:55–60.
8. Itani HA, Xiao L, Saleh MA, Wu J, Pilkinton MA, Dale BL, *et al.* CD70 exacerbates blood pressure elevation and renal damage in response to repeated hypertensive stimuli. *Circ Res* 2016; 118: 1233–1243.
9. Vianello E, Dozio E, Rigolini R, Marrocco-Trischitta MM, Tacchini L, Trimarchi S, *et al.* Acute phase of aortic dissection: a pilot study on CD40L, MPO, and MMP-1, -2, 9 and TIMP-1 circulating levels in elderly patients. *Immun Ageing* 2016; 13:9.
10. Le NT, Takei Y, Izawa-Ishizawa Y, Heo KS, Lee H, Smrcka AV, *et al.* Identification of activators of ERK5 transcriptional activity by high-throughput screening and the role of endothelial ERK5 in vasoprotective effects induced by statins and antimalarial agents. *J Immunol* 2014; 193:3803–3815.
11. Taylor AP, Yadlapati A, Andrei AC, Li Z, Clennon C, McCarthy PM, *et al.* Statin use and aneurysm risk in patients with bicuspid aortic valve disease. *Clin Cardiol* 2016; 39:41–47.
12. National Research Council. *The guide for the care and use of laboratory animals*, 8th ed. Washington, DC: The National Academies Press; 2011.
13. Ocsan RJ, Lai YN, Prabhu KV, Hambly BD, McLachlan CS. Chronic NG-nitro-L-arginine methyl ester (L-NAME) administration in C57BL/6J mice induces a sustained decrease in c-kit positive cells during development of cardiac hypertrophy. *J Physiol Pharmacol* 2013; 64: 727–736.
14. Kurata T, Kawai H, Miyazaki K, Kozuki M, Morimoto N, Ohta Y, *et al.* Statins have therapeutic potential for the treatment of Alzheimer's disease, likely via protection of the neurovascular unit in the AD brain. *J Neurol Sci* 2012; 322:59–63.
15. Ishizawa K, Izawa-Ishizawa Y, Yamano N, Urushihara M, Sakurada T, Imanishi M, *et al.* Nitrosonifedipine ameliorates the progression of type 2 diabetic nephropathy by exerting antioxidative effects. *PLoS One* 2014; 9:e86335.
16. Nagashima T, Shirakawa H, Nakagawa T, Kaneko S. Prevention of antipsychotic-induced hyperglycaemia by vitamin D: a data mining prediction followed by experimental exploration of the molecular mechanism. *Sci Rep* 2016; 6:26375.
17. Zhao S, Nishimura T, Chen Y, Azeloglu EU, Gottesman O, Giannarelli C, *et al.* Systems pharmacology of adverse event mitigation by drug combinations. *Sci Transl Med* 2013; 5:206ra140.
18. Alfieri A, Ong AC, Kammerer RA, Solanky T, Bate S, Tasab M, *et al.* Angiopoietin-1 regulates microvascular reactivity and protects the microcirculation during acute endothelial dysfunction: role of eNOS and VE-cadherin. *Pharmacol Res* 2014; 80:43–51.
19. Kurihara T, Shimizu-Hirota R, Shimoda M, Adachi T, Shimizu H, Weiss SJ, *et al.* Neutrophil-derived matrix metalloproteinase 9 triggers acute aortic dissection. *Circulation* 2012; 126:3070–3080.
20. Giani JF, Shah KH, Khan Z, Bernstein EA, Shen XZ, McDonough AA, *et al.* The intrarenal generation of angiotensin II is required for experimental hypertension. *Curr Opin Pharmacol* 2015; 21:73–81.
21. Kopincova J, Puzserova A, Bernatova I. L-NAME in the cardiovascular system – nitric oxide synthase activator? *Pharmacol Rep* 2012; 64: 511–520.
22. Rochette L, Lorin J, Zeller M, Guillard JC, Lorgis L, Cottin Y, *et al.* Nitric oxide synthase inhibition and oxidative stress in cardiovascular diseases: possible therapeutic targets? *Pharmacol Ther* 2013; 140: 239–257.
23. Albrecht EW, Stegeman CA, Heeringa P, Henning RH, van Goor H. Protective role of endothelial nitric oxide synthase. *J Pathol* 2003; 199:8–17.
24. Kanematsu Y, Yamaguchi K, Ohnishi H, Motobayashi Y, Ishizawa K, Izawa Y, *et al.* Dietary doses of nitrite restore circulating nitric oxide level and improve renal injury in L-NAME-induced hypertensive rats. *Am J Physiol Renal Physiol* 2008; 295:F1457–F1462.
25. Torrens C, Kelsall CJ, Hopkins LA, Anthony FW, Curzen NP, Hanson MA. Atorvastatin restores endothelial function in offspring of protein-restricted rats in a cholesterol-independent manner. *Hypertension* 2009; 53:661–667.
26. Perez-Guerrero C, Alvarez de Sotomayor M, Jimenez L, Herrera MD, Marhuenda E. Effects of simvastatin on endothelial function after chronic inhibition of nitric oxide synthase by L-NAME. *J Cardiovasc Pharmacol* 2003; 42:204–210.
27. Kabacik S, Raj K. Ionising radiation increases permeability of endothelium through ADAM10-mediated cleavage of VE-cadherin. *Oncotarget* 2017; 8:82049–82063.
28. Fischer A, Braga VMM. Vascular permeability: flow-mediated, noncanonical notch signalling promotes barrier integrity. *Curr Biol* 2018; 28:R119–R121.
29. Suzuki R, Nakamura Y, Chiba S, Mizuno T, Abe K, Horii Y, *et al.* Mitigation of tight junction protein dysfunction in lung microvascular endothelial cells with pitavastatin. *Pulm Pharmacol Ther* 2016; 38: 27–35.
30. Berry CL, Sosa-Melgarejo JA, Greenwald SE. The relationship between wall tension, lamellar thickness, and intercellular junctions in the fetal and adult aorta: its relevance to the pathology of dissecting aneurysm. *J Pathol* 1993; 169:15–20.